10/520696
DT15 Rec'd PCT/PTO 07 JAN 2005

TITLE OF THE INVENTION

Plant nucleotide sugar pyrophosphatase/phosphodiesterase (NPPase), method of production, use in the manufacture of testing devices and its application in the production of transgenic plants.

INDUSTRIAL FIELD TO WHICH THE INVENTION RELATES

The invention relates to the field of the production, purification and characterization of various isoforms of nucleotide pyrophosphatase/phosphodiesterase (NPPase) especially of rice and barley, and to the applications of this enzyme in the determination of levels of nucleotide sugars, and of sulphonucleotides, as well as in the production of transgenic plants in which there is overexpression of cDNA's of the genes that code for the said isoforms of NPPase, giving rise to plants with reduced content of starch and cell-wall polysaccharides and high resistance to salinity and temperature.

STATE OF THE PRIOR ART

Starch is the principal storage form of carbohydrates in plants. It is accumulated in large quantities in organs such as seeds (wheat, barley, maize, pea, etc.) and tubers (potato and yam among others), and is a fundamental constituent of the human diet. On the other hand, starch is a polymer that is often used in the paper, cosmetic, pharmaceutical and food industries, and is also used as a basic component for the manufacture of biodegradable plastics and environment-friendly paints. Another polysaccharide, cellulose, is a fundamental component of the cell wall of plants, constituting the fundamental raw material in such important processes as papermaking. Consequently, investigation of the processes involved in the synthesis of these polymers of glucose is given priority in various areas of industrial production.

UDPglucose (UDPG) is the fundamental precursor of the biosynthesis of cellulose and of cell-wall polysaccharides. It is also the precursor molecule of processes connected with the glycosylation of proteins and lipids. On the other hand, ADPglucose (ADPG) is the universal precursor of the biosynthesis of starch in storage tissues of plants. Its concentration in the cell is determining for the quantity and quality of the starch produced by the plant. Considerations of the factors that govern the endogenous levels of ADPG and UDPG in the plant cell have basically revolved around their synthesizing enzymes, such as ADPG pyrophosphorylase, UDPG pyrophosphorylase and sucrose synthase (Preiss, (1988) "Biosynthesis of starch and its regulation". The Biochemistry of Plants. Vol. 14, Academic Press, New York, pp. 182-249; Pozueta-Romero, J., Perata, P., Akazawa, T. (1999) "Sucrose-starch conversion in heterotrophic tissues". Crit. Rev. Plant Sci. 18, 489-525). However, there has been little study of the mechanisms responsible for the degradation of these nucleotide sugars (Feingold, D.S., Avigad, G. (1980) "Sugar transformation in plants". The Biochemistry of Plants. Vol. 3, Stumpf, P.K. and Conn, E.E. eds. Academic Press, New York, pp. 101-170). There are indications that both bacteria and mammals possess enzymatic mechanisms capable of hydrolysing ADPG and UDPG (Melo, A., Glaser, L. (1966) "Nucleotide diphosphate hexose pyrophosphatases". Biochem. Biophys. Res. Commun. 22, 524-531; Bessman, M.J., Frick, D.N., O'Handley, S.F. (1996) "The MutT proteins or Nudix hydrolases, a family of versatile, widely distributed housecleaning enzymes". J. Biol. Chem. 271, 25059-25062; Rodríguez, P., Bass, S.T., Hansen, R.G. (1968) "A pyrophosphatase from mammalian tissues specific for derivates of ADP". Biochim. Biophys. Acta, 167, 199-201; Gasmi, L., Cartwright, J.L., McLennan, A.G. (1999) "Cloning, expression and characterization of YSA1H, a human adenosine 5'-diphosphosugar pyrophosphatase possessing a MutT motif". Biochem. J. 331-337; Moreno-

Bruna, B., Baroja-Fernández, E., Muñoz, F.J., Bastarrica-Berasategui, A., Zandueta-Criado, A., Rodríguez-López, M., Akazawa, T., Pozueta-Romero, J. (2001) "Adenosine diphosphate sugar pyrophosphatase prevents glycogen biosynthesis in Escherichia coli". Proc. Natl. Acad. Sci. 98, 8128-8132). In plants, activity of this kind has barely been described in the literature (Salvucci, M.E., Crafts-Brandner, S.J. (1995) "Purification and properties of a unique nucleotide pyrophosphatase/phosphodiesterase I that accumulates in soybean leaves in response to fruit removal". Plant Physiol. 108, 1269-1276; Rodríguez-López, M., Baroja-Fernández, E., Zandueta-Criado, A., Pozueta-Romero, J. (2000) "Adenosine diphosphate glucose pyrophosphatase: a plastidial phosphodiesterase that prevents starch biosynthesis". Proc. Natl. Acad. Sci., 97, 8705-8710; Baroja-Fernández, E., Zandueta-Criado, A., Rodríguez-López, M., Akazawa, T., Pozueta-Romero, J. (2000) "Distinct isoforms of ADPglucose pyrophosphatase and ADPglucose pyrophosphorylase occur in the suspension-cultured cells of sycamore (*Acer pseudoplatanus* L.). FEBS Lett. 480, 277-282; Rodríguez-López, M., Baroja-Fernández, E., Zandueta-Criado, A. Moreno-Bruna, B. Muñoz, F.J., Akazawa, T., Pozueta-Romero, J. (2001) "Two isoforms of a nucleotide-sugar pyrophosphatase/phosphodiesterase from barley leaves (*Hordeum vulgare* L.) are distinct oligomers of HvGLP1, a germin-like protein". FEBS Lett. 490, 44-48).

In various industries, starch is a basic essential as a viscosity-increasing and gelling agent. The biosynthesis of starch in the plant cell starting from ADPG takes place in the subcellular compartment called the plastid. ADPG is both synthesized and utilized in this compartment, and therefore the levels of starch can be controlled by controlling the processes that regulate the ADPG levels. The various applications of starch produced in a plant are based on the balance of amylose and amylopectin, which determines the structure of the starch granule, as well as its viscosity in

aqueous suspensions. The ratio of amylose to amylopectin depends on the concentration of ADPG in the plant cell. To date, no method is known for regulating the characteristics of the starch produced in a plant by controlling the degradation of ADPG, which the enzyme described in the present invention, can provide.

In addition to acting as a storage substance for the plant, starch accumulates in the plant cell in circumstances where the plant is not subject to conditions of water stress. In conditions where the plant is subjected to high temperatures or high concentrations of salts in the environment, the plant stops accumulating starch, and produces large quantities of soluble sugars that accumulate in the vacuole (Keeling, P.L., Bacon, P.J., Holt, D.C. (1993) "Elevated temperature reduces starch deposition in wheat endosperm by reducing the activity of soluble starch synthase" Planta 191, 342-348; Geigenberger, P., Geiger, M., Stitt, M. (1998) "High-temperature perturbation of starch synthesis is attributable to inhibition of ADP-glucose pyrophosphorylase by decreased levels of glycerate-3-phosphate in growing potato tubers" Plant Physiol. 117, 1307-1316). In addition to these adaptive changes of carbohydrate metabolism to water stress, the plant undergoes changes in its sulphur metabolism, avoiding the accumulation of adenosine-5'-phosphate (PAP) arising from the transformation of adenosine 5'-phosphosulphate (APS) and 3'-phosphoadenosine 5'-phosphosulphate (PAPS) (Gil-Mascarell, R., López-Coronado, J.M., Bellés, J.M., Serrano, R., Rodríguez, P.L. (1999) "The Arabidopsis HAL2-like gene family includes a novel sodium-sensitive phosphatase" Plant J. 17, 373-383). On the basis of these observations, it is possible that enzymatic reactions responsible for the hydrolysis of ADPG, APS and PAPS are responsible for adaptive processes of the plant to the conditions of water stress.

Chromatographic and radiological techniques are powerful tools for determining levels of nucleotides

such as sulphonucleotides (APS and PAPS among others; Yoshida, H., Fukui, S., Yamashina, I., Tanaka, T., Sakano, T., Usui, T., Shimotsuji, T., Yabuuchi, H., Owada, M., Kitagawa, T. (1982) "Elevation of nucleotide pyrophosphatase activity in skin fibroblasts from patients with Lowe's syndrome". Biochem. Biophys. Res. Commun. 107, 1144-1150) and nucleoside diphosphate sugars (such as the derivatives of glucose, ribose, mannose, galactose, glucuronic acid, fructose and galacturonic acid) in raw extracts of animal, plant or microbial origin. Although of very general use, they require a considerable investment in equipment and in the preparation of the test samples. Unfortunately, apart from a few exceptions (Puhakainen, E., Saarinen, A., Hänninen, O. (1977) "UDPglucuronic acid pyrophosphatase assay with the aid of alkaline phosphatase" Acta Chem. Scandinavica B31, 125-129) scant use is made of possible alternative methods that permit the detection and quantification of nucleotide sugars and sulphonucleotides in a simple and efficient manner. Analysis of the blood, muscle, kidney and liver levels of some of the aforementioned nucleotide sugars is important in clinical practice (Cortes, P., Dumler, F., Sastry, K.S., Verghese, C.P., Levin, N.W. (1982) "Effects of early diabetes on uridine diphosphosugar synthesis in the rat renal cortex". Kidney Int. 21, 676-682; Spiro, M.J. (1984) "Effect of diabetes on the sugar nucleotides in several tissues of the rat" Diabetologia 26, 70-75; Sochor, M., Kunjara, S., Baquer, N.Z., McLean, P. (1991) "Regulation of glucose metabolism in livers and kidneys of NOD mice". Diabetes 40, 1467-1471). For example, since UDPG is the precursor of glycogen in animals, analysis of the levels of this molecule can be important in the investigation and diagnosis of diseases associated with carbohydrate metabolism, such as various types of diabetes. On the other hand, determination of the levels of PAPS in the urine is essential for the diagnosis of serious illnesses such as Lowe's syndrome

or antiphospholipid syndrome (Yoshida, H., Fukui, S., Yamashina, I., Tanaka, T., Sakano, T., Usui, T., Shimotsuji, T., Yabuuchi, H., Owada, M., Kitagawa, T. (1982) "Elevation of nucleotide pyrophosphatase activity in skin fibroblasts from patients with Lowe's syndrome". Biochem. Biophys. Res. Commun. 107, 1144-1150; Amigo, M.C., García-Torres, T. (2000) "Morphology of vascular, renal, and heart lesions in the antiphospholipid syndrome: relationship to pathogenesis" Curr. Rheumatol. Rep. 2000, 2, 262-270). Obviously the possibility of analysing, simply and inexpensively, the levels of these substances in a sample represents an advantageous alternative to the chromatographic techniques.

The invention describes the purification and applications of an enzymatic product of plant origin that we designate NPPase, which catalyses the hydrolysis of small molecules with phosphodiester or phosphosulphate bonds and in particular ADPG and APS, as the preferred substrates. In the first priority (ES 200201647), with the experimental data available to them at that time, the inventors tentatively designated the enzymatic product isolated as NSPPase, but in the later, second priority (ES 200202673), with the data that had been accumulated, its designation was changed to NPPase as at present.

The plant enzyme of the invention has various isoforms in the plant tissues from which it can be obtained (Baroja-Fernández, E., Zandueta-Criado, A., Rodríguez-López, M., Akazawa, T., Pozueta-Romero, J. (2000) "Distinct isoforms of ADPglucose pyrophosphatase and ADPglucose pyrophosphorylase occur in the suspension-cultured cells of sycamore (*Acer pseudoplatanus* L.). FEBS Lett. 480, 277-282). The isoform that is simplest to extract is the one we call soluble, whereas other isoforms, which we can call particulates, are found firmly adhering to the starch granules.

In the present invention we succeeded in purifying and partially sequencing various soluble isoforms of NPPase

of barley and rice with approximate sizes of 70 kDa. On the basis of these sequences, we were able to isolate the cDNA's that code for the NPPases. After comparing their sequences with those available in databases, it was observed that the NPPases of rice and barley share homology with PPD1, a nucleotide phosphatase/ phosphodiesterase of *Lupinus luteus* which, in contrast to the NPPase of this invention, has as its best substrates the di- and tri-phosphate nucleosides but does not hydrolyse nucleotide sugars (Olczak, M., Olczak, T. (2002) "Diphosphonucleotide phosphatase/phosphodiesterase from yellow lupin (*Lupinus luteus* L.) belongs to a novel group of specific metallophosphatases". FEBS Lett. 519, 159-163). Moreover, it shares homology with other sequences that code for unknown or possible proteins of dicotyledonous plants such as Arabidopsis and chickpea.

One object of the invention is, firstly, the production, characterization and sequencing of various soluble 70-kDa isoforms of NPPase in substantially pure form, starting from plant tissues of barley (*Hordeum vulgare*) and rice (*Oryza sativa*). Another object is the production of complete cDNA's that code for the NPPases and contrasting them with sequences available in databases. The design of constructions derived from the cDNA's of the soluble NPPases intended for the production of transgenic plants with high NPPase activity whose content and quality of the starch, as well as that of cell-wall polysaccharides, are modified relative to control plants, is detailed later. The said plants do not accumulate the marker of osmotic toxicity PAP, so that they are more resistant to high concentrations of salts than the control plants.

Another object of the invention is the method used for making devices or kits for determination of nucleoside diphosphate sugars and sulphonucleotides based on the use of the enzymatic product with NPPase activity.

DETAILED DESCRIPTION OF THE INVENTION

The plant product with NPPase enzyme activity according to the invention can be obtained and purified starting from any plant tissue from any species, i.e. any monocotyledon or dicotyledon, for example barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), rice (*Oryza sativa*), pepper (*Capsicum annuum*), tomato (*Lycopersicon sculentum*), potato (*Solanum tuberosum*), Arabidopsis (*Arabidopsis thaliana*), or sycamore (*Acer pseudoplatanus* L.), to cite just some of the numerous representative examples of different families and genera.

Production and purification of a soluble isoform of NPPase:

The general method for obtaining and purifying the soluble plant NPPase described in the invention includes the following steps, in which slight variations are admissible that do not substantially alter the general scheme of the method of extraction and purification. NPPase is especially abundant in young leaves and is practically absent from storage tissues such as endosperms of seeds and tubers, accordingly it is recommended to use young leaves for the extraction of NPPase.

1. Homogenization of the plant tissue with an extraction buffer.
2. Filtration through four layers of Miracloth® (filter cloth for milk whey used in cheesemaking).
3. Ultracentrifugation of the filtered homogenate.
4. Precipitation of the proteins of the supernatant in ammonium sulphate.
5. Resuspension of the precipitate in buffer with pH of 4.2.
6. Heating for at least 15 minutes at a temperature between 60 and 65°C.

7. Centrifugation.

8. Concentration of the supernatant and purification of the protein by gel-filtration chromatography. The enzyme activity of NPPase is detected by detecting the production of G1P and AMP in samples incubated with substrates such as UDPG or ADPG.

9. Application of the NPPase to affinity column chromatography of the Concanavalin A type, which indicates that the NPPase is glycosylated.

10. Isoelectric focusing in a Multiphor II system, using PAGplates with pH range between 3.5 and 9.5 (Amersham/Pharmacia). The position of the NPPase can be determined easily in one of the following ways:

a) Elution of the protein followed by detection of the production of G1P in the presence of ADPG or UDPG.

b) Incubation of the gel in a solution with bis-paranitrophenylphosphate (bis-PNPP) and development in a basic solution as described by Nishimura and Beevers (Nishimura, M., Beevers, H. (1978) Plant Physiol. 62, 44-48).

11. Separation of the protein in denaturing gel by electrophoresis in a system of neutral or slightly acid buffers such as NuPAGE 4-12% Bis-Tris (Novex, San Diego, California). The position of the NPPase can be determined easily in one of the following ways:

a) Elution of the protein followed by detection of the production of G1P in the presence of ADPG.

b) Incubation of the gel in a solution with bis-PNPP and development in a basic solution.

Identification of the product with NPPase enzyme activity

The enzyme product obtained by the methods described above, or other equivalent methods, is identified by means of the following functional standards:

- It is a pyrophosphatase/phosphodiesterase that catalyses the hydrolysis of ADPG in equimolar quantities of G1P and AMP (Rodríguez-López, M.,

Baroja-Fernández, E., Zandueta-Criado, A., Pozueta-Romero, J. (2000) "Adenosine diphosphate glucose pyrophosphatase: a plastidial phosphodiesterase that prevents starch biosynthesis". Proc. Natl. Acad. Sci., 97, 8705-8710)

- In addition to ADPG, it recognizes small molecules that possess phosphodiester and phosphosulphate bonds, such as UDPG, GDP-glucose, GDP-mannose, ADP-mannose, bis-PNPP, PAPS and APS and others of similar structure.
- It does not hydrolyse molecules with phosphomonoester bonds such as G1P, G6P, AMP, 3-phosphoglycerate, and other similar ones. Nor does it hydrolyse cyclic AMP or long-chain nucleic acids such as DNA or RNA, which are substrates of other phosphodiesterases described in the literature.
- In contrast to pyrophosphatases of ADP-sugars (EC 3.6.1.13, EC 3.6.1.21) described in bacteria and animals and in contrast to other phosphodiesterases (EC 3.1.4), its ionic requirements are reduced, therefore it can work in the absence of ions of magnesium, manganese, cobalt, and other divalent cations.
- In contrast to the pyrophosphatases of nucleoside diphosphate sugars of bacteria and animals, NPPase hydrolyses bis-PNPP.
- It is inhibited by phosphorylated molecules such as AMP, ADP, ATP, 3-phosphoglycerate, orthophosphate, inorganic pyrophosphate, and others with similar characteristics.
- It is strongly inhibited by molybdate and arsenate.
- It is resistant to ionic detergents such as SDS (sodium dodecylsulphate).
- It is resistant to the action of a wide range of proteases, such as Proteinase K and Pronase (Boehringer).
- Its activity is not affected by the action of typical inhibitors of phosphodiesterases such as β-

mercaptoethanol, EDTA, reduced cysteine, ascorbate, and other reducing and chelating agents.

- It is sensitive to slightly basic pH and is very stable at pH between 4 and 7.5.

Production of a complete cDNA that codes for the soluble NPPase

Some known internal amino acid sequences of the various NPPases purified were compared with others present in the databases. The result of this analysis made it possible to design two primers that were used for obtaining, by RT-PCR, a cDNA that codes for an NPPase of rice and of barley. The cDNA's obtained were cloned in the pGEM-T vector and were used as probes for searching for a complete cDNA in the cDNA library of young rice leaves. The complete cDNA obtained was introduced in the EcoRV restriction site of the plasmid pSK Bluescript (Stratagene) giving rise to the construction pNPP (Fig. 1) which was amplified in the host bacterium XL1 Blue. A strain of this bacterium was deposited on 15.10.02 in the Spanish Type Culture Collection, University of Valencia, Research Building, Campus of Burjasot, 46100 Burjasot, Valencia, with the number CECT 5739.

Production of transgenic plants that overexpress the cDNA of soluble NPPase

pNPP was digested successively with the enzymes HindIII, T4 DNA polymerase and XbaI. The fragment released (which contains the cDNA of NPPase) was cloned in the plasmid pVT'BSP after being digested successively by the enzymes NcoI, T4 DNA polymerase and XbaI. In this way we obtain a plasmid designated p35SNPPNOS which has the constitutive promoter 35S, the cDNA of NPPase and the Nos terminator.

To be able to transfer this construction to the genome of the plants via *Agrobacterium tumefaciens*, it is

necessary for it to be cloned previously in a binary plasmid. For this, p35SNPPNOS was digested successively with the enzymes HindIII, T4 DNA polymerase and XbaI and was cloned within the binary plasmid pBIN20 (Hennegan, K.P., Danna, K.J. "pBIN20: An improved binary vector for Agrobacterium-mediated transformation" Plant Mol. Biol. Rep. 16, 129-131) which had previously been digested successively with the enzymes HindIII, T4 DNA polymerase and XbaI. The plasmid thus obtained was designated with the name of pBIN20-35S-NPP (Fig. 2). After amplification in E. coli (XL1 Blue), pBIN20-35S-NPP was introduced into *Agrobacterium tumefaciens*, which was used for transforming species such as tomato, tobacco, potato etc. (Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., Fraley, R.T. (1985) "A simple and general method for transferring genes into plants" Science 277, 1229-1231. The strain of *Agrobacterium tumefaciens* was deposited in the Spanish Type Culture Collection on 16.05.2003, located in the Research Building of the University of Valencia, Campus of Burjasot, Burjasot 46100 (Valencia, Spain) with the deposition number CECT 5799.

Preparation of devices (assay kits) for determination of nucleoside diphosphate sugars and sulphonucleotides

The kits designed for the determination of nucleotides such as nucleoside diphosphate sugars and sulphonucleotides are based on the action of the product with NPPase activity on phosphodiester and phosphosulphate bonds of small molecules which, after being hydrolysed, give rise to other molecules that are easy to detect and quantify.

The two most suitable strategies for making these kits start from the hydrolysis of the nucleoside diphosphate sugar by the enzyme according to the present invention, i.e. NPPase, producing equimolar quantities of sugar-1-phosphate and of the corresponding nucleoside

monophosphate. From here on, consideration can be given to determination of the quantity of nucleotide initially present in the sample based on determination of the quantity of sugar-1-phosphate and nucleoside monophosphate produced, as specified below:

- In the case when the sugar-1-phosphate is glucose-1-P (G1P), the said compound is submitted to the action of the enzyme phosphoglucomutase yielding glucose-6-phosphate, which in its turn can be made to undergo a coupling reaction with NAD^+ by action of the enzyme glucose-6-phosphate dehydrogenase, obtaining 6-phosphogluconate and NADH, which is easily determined.
- In the case when the sugar-1-phosphate is not G1P, its determination and that of the nucleoside monophosphate take place by colorimetric determination of the orthophosphate (Pi) produced after the hydrolysis of these compounds with alkaline phosphatase. Alternatively, as the coupling enzyme it is possible to use 5'-nucleotidase, which will hydrolyse the nucleoside monophosphate to equimolar quantities of the corresponding nucleoside and Pi. The Pi released in either of the two cases is easily quantifiable by known colorimetric methods.

The strategy for determination of levels of sulphonucleotides such as APS is based on the hydrolysis of these nucleotides and consequent production of equimolar quantities of sulphate, which can be determined by turbidimetry or by nephelometry (Sörbo, B. (1987) "Sulfate: turbidimetric and nephelometric methods" Methods Enzymol. 143, 3-6).

Production of polyclonal antibodies specific to plant NPPase

Two milligrams of purified NPPase were separated in SDS-PAGE. After being eluted, it was mixed with complete Freund's adjuvant (at 50/50 ratio) and was then aliquoted in three equal fractions, each of which was injected into a rabbit at intervals of two weeks.

Approximately three months after the first injection, the blood serum of the rabbit was extracted, which contains the polyclonal antibodies specific to AGPPase.

Identification of the product by Western blotting

Samples of proteins from wild plants and transgenic plants that overexpress the gene that codes for NPPase of rice were separated by SDS-PAGE. Then they were transferred to nitrocellulose membranes and the NPPase was detected using the specific anti-NPPase antibody according to the methodology described in the literature (Towbin, H., Staehelin, T., Gordon, J. (1979) "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications" Proc. Natl. Acad. Sci. USA 76, 4350-4354.

EXAMPLES OF APPLICATION OF THE INVENTION

Examples are presented below which describe in detail the method of production and purification of the soluble NPPase of young barley leaves. The same method, with slight changes appropriate to each case, can be applied to any other species. Other examples describe the use of NPPase for the production of assay kits for determination of nucleotide sugars and sulphonucleotides. Another example shows the production of a complete cDNA that codes for soluble NPPase.

Example 1: Extraction and purification of the soluble NPPase obtained from barley leaves

All the steps were carried out at 4°C, unless indicated otherwise. The plant tissue (900 g) was homogenized with 2900 mL of extraction buffer (Mes 50 mM pH 6, EDTA 1 mM, DTT 2 mM) using a Waring blender. The homogenate was filtered through four layers of Miracloth, centrifuged at 100 000 *g* for 30 minutes and the supernatant was adjusted to 50% of ammonium sulphate.

The precipitate obtained after 30 minutes of centrifugation at 30 000 *g* (20°C) was resuspended in 2900 mL of Mes 50 mM pH 4.2, then heated on a water bath at 62°C for 20 minutes, cooled in ice, and centrifuged at 30 000 *g* for 20 minutes. The proteins of the supernatant were precipitated using 50% ammonium sulphate, and resuspended in 5.7 mL of Mes 50 mM pH 6. Then the sample was submitted to gel filtration in a Superdex 200 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) pre-equilibrated with Mes pH 6 and NaCl 150 mM. The fractions with NPPase activity were combined, concentrated, and submitted to isoelectric focusing in a native gel, permitting purification of the protein to homogeneity (Fig. 3).

Example 2: Enzymatic tests

Unless stated otherwise, all the enzymatic reactions took place at 37°C. The determinations of NPPase activities were performed using spectrophotometric determination of G1P in two steps described by Sowokinos (1981) (Sowokinos, 1981, Plant Physiol. 68, 924-929). The reaction mixture contained Hepes 50 mM pH 7, the specified quantity of ADPG and the protein extract in a total volume of 50 microlitres. All the assays were performed relative to an ADPG blank. After 20 minutes of incubation, the reaction was stopped by boiling in a dry bath for 2 minutes. The mixture was centrifuged at 20 000 *g* for 5 minutes and the supernatant was recovered. In the second step, G1P was determined spectrophotometrically in 300 microlitres of mixture containing Hepes 50 mM pH 7, EDTA 1 mM, $MgCl_2$ 2 mM, KCl 15 mM, NAD^+ 0.6 mM, one unit of phosphoglucomutase and one unit of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, and 30 microlitres of the supernatant resulting from step 1. After 20 minutes of incubation, the production of NADH was monitored at 340 nm using a Multiskan EX spectrophotometer (Labsystems). The amount of NADH

produced by any protein extract in the absence of ADPG in step 1 was negligible.

The native molecular weight of the NPPase was determined by gel filtration by plotting the partition coefficient (Kav) against the logarithm of the molecular weight of the following protein standards: bovine thyroglobulin (670 kDa), bovine gamma-globulin (158 kDa), ovalbumin (45 kDa), myoglobin (17 kDa) and vitamin B-12 (1.3 kDa). The protein content was determined by Bradford's method using the reagent made by Bio-Rad and gamma-globulin as standard.

Table 1 presented below shows the purification of soluble NPPase starting from barley leaves. The unit (U) is defined as the quantity of enzyme that catalyses the production of 1 µmol of product per minute.

Table 1

	Total volume (mL)	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg protein)	Purification (factor)
Supernatant 100000 x *g*	2900	24 286	1 195 000	50	-
pH 4.2 / 62°C	2900	778	1 067 000	1370	28
Superdex 200	86	164	300	1820	37
Isoelectric focusing	14	0.6	21 870	36 455	740

Example 3: Identification of the product with enzymatic activity obtained

The product with NPPase activity thus obtained complies with the following characteristics:

- It catalyses the hydrolysis of ADPG producing equimolar quantities of G1P and AMP.

- In addition to ADPG, the NPPase recognizes other small molecules that possess phosphodiester bonds, such as UDPG, GDP-glucose, bis-PNPP and others of similar structure. It also catalyses the hydrolysis of small molecules with phosphosulphate bonds, such as APS, releasing equimolar quantities of sulphate and AMP (Table 2 -Vmax in % in relation to ADPG- and Table 3).
- It does not hydrolyse molecules with phosphomonoester bonds such as G1P, G6P, AMP, 3-phosphoglycerate, and other similar molecules. Nor does it hydrolyse cyclic AMP or nucleic acids such as DNA and RNA, which are substrates of other phosphodiesterases described in the literature.
- Its ionic requirements are reduced, therefore the NPPase can work in the absence of ions of magnesium, manganese, cobalt, and other divalent cations, which are essential effectors for the functioning of other phosphodiesterases described in the literature.
- In contrast to the pyrophosphatases of nucleoside diphosphate sugars of bacteria and animals, NPPase hydrolyses bis-PNPP.
- It is inhibited by phosphorylated molecules such as AMP, ADP, ATP, 3-phosphoglycerate, orthophosphate, inorganic pyrophosphate, and others with similar characteristics.
- It is strongly inhibited by molybdate and arsenate.
- It is resistant to ionic detergents such as SDS (sodium dodecylsulphate).
- It is resistant to the action of a wide range of proteases, such as Proteinase K and Pronase (Boehringer).
- Its activity is not affected by the action of typical inhibitors of phosphodiesterases such as β-mercaptoethanol, EDTA, reduced cysteine, ascorbate, and other reducing and chelating agents.
- It is sensitive to slightly basic pH and is very stable at pH between 4 and 7.5. This characteristic is one that makes NPPase a completely different enzyme from the majority of the phosphodiesterases described

in the literature, as the latter are stable and active at slightly basic pH values.

- It withstands a temperature of 65°C for 30 minutes, and can be characterized by the following data:
- Apparent molecular weight measured by gel filtration, around 70 kDa and 270 kDa, from which it is deduced that it has a monomeric form of 70 kDa and another homopolymeric form.
- K_{eq}' of the reaction 110.
- Increase in standard free energy ($\Delta G'$) of -2.9 kcal/mol.
- It is a glycoprotein, since it is retained by columns of concanavalin.
- Apparent molecular weight of the protein purified in denaturing gels, around 70 kDa.
- The amino acid sequences of barley NPPase obtained are:
  - N-terminal end: SEQ ID NO: 1
  - Internal sequences (obtained after partial hydrolysis of NPPase with trypsin): SEQ ID NO: 2, 3, 4, 5 and 6.
- The amino acid sequences of rice NPPase obtained are:
  - N-terminal end: SEQ ID NO: 7
  - Internal sequences (obtained after partial hydrolysis of NPPase with trypsin): SEQ ID NO: 8, 9, 10, 11, 12, 13, 14, 15, 16 and 17.

Table 2: Kinetic parameters and substrate specificity of NPPase from barley leaves

substrate	Km (mM)	Vmax
ADPG	0.5	100
ATP	3.5	40
ADP	3.5	40
APS	0.5	160
PAPS	-	-
PAP	n.q.	n.q.
ADPmannose	0.4	30
GDPmannose	0.4	30
CDPG	2.8	114
UDPG	2.1	114
ADPribose	2.4	100
NAD^+	2.5	100
$NADP^+$	n.q.	n.q.
bis-PNPP	0.3	100
PNPP	0.5	100
Hexose monophospates	n.q.	n.q.
Nucleotide monophosphates	n.q.	n.q.

Substrate	K_m (mM)	V_{max} (μmol min^{-1} (mg protein)$^{-1}$)	k_{cat}/K_m (M^{-1} s^{-1})
ADP-glucose	0.60	183	3.50×10^5
ADP-ribose	1.43	317	2.48×10^5
UDP-glucose	1.47	242	1.93×10^5
CDP-glucose	0.92	107	1.93×10^5
GDP-glucose	0.93	119	1.48×10^5
GDP-mannose	0.80	129	1.90×10^5
TDP-glucose	0.60	106	2.06×10^5
bis-PNPP	1.30	191	1.71×10^5
ATP	2.06	356	2.03×10^5
ADP	4.33	385	1.00×10^5
AMP	n.d.	n.d.	-
PPi	-	176	-
APS	3.04	282	1.09×10^5

Table 3: Kinetic parameters and substrate specificity of the NPPase from rice leaves

Example 4: Obtaining a complete cDNA that codes for the soluble NPPase from rice and an incomplete cDNA that codes for the soluble NPPase of barley

Knowledge of internal sequences of rice NPPase made it possible to design primers which, in the direction 5' - 3', are SEQ ID NO: 18 and 19. Using this primer, a cDNA was amplified by conventional methods of RT-PCR and was used as a probe for obtaining cDNA's from cDNA libraries from young leaves of rice and barley. As a result, a complete cDNA of rice NPPase was obtained and was inserted in a pSK Bluescript plasmid (Stratagene) which was amplified in the host bacterium XL1 Blue. The sequence of the complete cDNA of rice NPPase is SEQ ID NO: 20 and the amino acid deduced is SEQ ID NO: 21. An incomplete cDNA of barley NPPase was also obtained (SEQ

ID NO: 22) and its deduced amino acid sequence is SEQ ID NO: 23. For this, the primer represented by SEQ ID NO: 24 was eliminated. After comparing with sequences available in databases, it was observed that the rice NPPase possesses 60% homology with PPD1 (access No. AJ421009), a nucleotide phosphatase/phosphodiesterase of *L. luteus* that hydrolyses nucleoside di- and triphosphates but is unable to hydrolyse nucleotide sugars (Olczak, M., Olczak, T. (2002) "Diphosphonucleotide phosphatase/phosphodiesterase from yellow lupin (*Lupinus luteus* L.) belongs to a novel group of specific metallophosphates". FEBS Lett. 519, 159-163). Moreover, as shown in Fig. 4, cDNA's were sequenced for dicotyledonous plants such as Arabidopsis (access No. AY099570) and chickpea (access No. AJ271664) which code for unknown or possible proteins that show high homology with the rice NPPase.

Example 5: Products with NPPase activity from various plants

The NPPase enzyme exhibits a very wide distribution among plants, so that the enzymatic product with NPPase activity can be obtained from any plant, especially from tissues of young plants such as leaves and roots. For example, the following Table 4 shows the specific activities (mU / mg protein) obtained in various monocotyledons and dicotyledons.

Table 4

	Specific activity (mU / mg protein)
	(+ADPG)
Monocotyledons	
Leaf of barley (*Hordeum vulgare*)	113.7 ± 3.5
Leaf of wheat (*Triticum aestivum*)	22.4 ± 2.5
Dicotyledons	

Leaf of *Arabidopsis thaliana* (Wt)	5.2 ± 0.6
Leaf of pepper (*Capsicum annuum*)	5.0 ± 0.6
Leaf of tomato (*Lycopersicon sculentum*)	5.6 ± 0.5
Cell culture of sycamore (*Acer pseudoplatanus*)	16.5 ± 7.2

Example 6: Preparation of enzyme kits for determination of glucose-nucleoside diphosphates

For the determination of glucose-nucleoside diphosphates such as ADPG, UDPG, CDP-glucose, GDP-glucose and TMP-glucose, a kit is prepared containing the following elements:

a. NPPase
b. NAD
c. Phosphoglucomutase (PGM)
d. G6P dehydrogenase (G6PDH)
e. Buffer

Determination of the quantity of glucose-nucleoside diphosphate present in the test sample is based on spectrophotometric determination of the NADH produced according to the following coupled reaction:

$$\text{(test sample)}\quad \text{NDP-glucose} \xrightarrow{\text{NPPase}} \text{NMP} + \text{G1P} \xrightarrow{\text{PGM}} \text{G6P} \xrightarrow[\text{G6PDH}]{\text{NAD}^+} \text{6-phosphogluconate} + \text{NADH}$$

The quantity of NDP-glucose in a test sample could be determined by preparing a cocktail with the composition (for 1 ml):

- Test sample
- 1 U of NPPase
- 1 U of PGM
- 1 U of G6PDH
- 0.6 mM NAD
- Buffer Mes or Hepes 50 mM pH 7

- Water (make up to 1 ml)

Incubate at 37°C for 20 minutes and observe the change in absorbance of the sample at 340 nm. A cocktail not containing NPPase can be used as a negative control.

Example 7: Preparation of enzyme kits for determination of nucleoside diphosphates of sugars other than glucose

Kits are prepared for determination of the following nucleoside diphosphate sugars:

- ribose-nucleoside diphosphates (ADP-ribose, GDP-ribose, UDP-ribose, CDP-ribose or TDP-ribose)
- mannose-nucleoside diphosphates (ADP-mannose, GDP-mannose, TDP-mannose, UDP-mannose or CDP-mannose)
- galactose-nucleoside diphosphates (ADP-galactose, GDP-galactose, UDP-galactose or CDP-galactose)
- glucuronic-nucleoside diphosphates (GDP-glucuronic, UDP-glucuronic, ADP-glucuronic, CDP-glucuronic or TDP-glucuronic)
- fructose-nucleoside diphosphates (GDP-fructose, ADP-fructose, CDP-fructose, UDP-fructose, TDP-fructose)
- galacturonic-nucleoside diphosphates (UDP-galacturonic, GDP-galacturonic, CDP-galacturonic, TDP-galacturonic or ADP-galacturonic)

The kit has the following elements:

a. NPPase
b. 5'-nucleotidase (or, alternatively, alkaline phosphatase)
c. buffer

Determination of the quantity of nucleoside diphosphate sugar present in the test sample is based on colorimetric determination of the orthophosphate released according to the following coupled enzymatic reaction:

(test sample)

NDP-sugar → sugar-P + NMP → base + Pi

NPPase 5'-nucleotidase

The Pi is determined according to any of the numerous colorimetric methods available in the bibliography and on the market.

The quantity of NDP-sugar in a test sample could be determined by preparing a cocktail (1 ml) consisting of:

- Test sample
- 1 U of NPPase
- 1 U of 5'-nucleotidase (or, alternatively, 1 U of alkaline phosphatase)
- Buffer Mes or Hepes 50 mM pH 7.5
- Water (make up to 1 ml)

Incubate at 37°C for 20 minutes and determine the production of Pi released according to conventional methods. A cocktail not containing NPPase can be used as negative control.

Example 8: Preparation of enzyme kits for determination of PAPS and APS

The strategy for determination of levels of sulphonucleotides such as PAPS or APS is based on turbidimetric or nephelometric determination according to the following reaction:

NPPase

PAPS → PAP+sulphate

NPPase

APS → AMP+sulphate

The quantity of sulphonucleotide in a test sample could be determined by preparing a cocktail (1 ml) consisting of:

- Test sample
- 1 U of NPPase
- Buffer Mes or Hepes 50 mM pH 7.0
- Water (make up to 1 ml)

Incubate at 37°C for 20 minutes and determine the production of sulphate released according to conventional methods. A cocktail not containing NPPase can be used as negative control.

Example 9: Production of transgenic plants of tobacco, potato and tomato that overexpress NPPase

Using the strain of *Agrobacterium tumefaciens* CECT 5799, we obtained plants of tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*) and tomato (*Lycopersicon sculentum*) with high NPPase activity in all the organs analysed (root, leaf, fruit and stalk) (Fig. 5). These plants accumulated large amounts of a protein that was recognized specifically by the polyclonal antibody obtained relative to rice NPPase (Fig. 6) and had the following characteristics:

1. Low content of starch and cell-wall carbohydrates (according to the measurement techniques based on commercial kits described in the literature (Frehner, M., Pozueta-Romero, J., Akazawa, T. (1990) "Enzyme sets of glycolysis, gluconeogenesis, and oxidative pentose phosphate pathway are not complete in nongreen highly purified amyloplasts of sycamore cell suspension cultures" Plant Physiol. 94, 538-544)).
2. High content of soluble sugars such as sucrose, glucose-6-phosphate, glucose and fructose.
3. Reduction of the levels of PAP accumulated in the tissues, imparting great resistance to high

concentrations of sodium chloride in the growth substrate, relative to untransformed plants.

4. Resistance to high temperatures.

DESCRIPTION OF THE DIAGRAMS:

Fig. 1: Schematic diagram of pNPP

Fig. 2 A-C: Production of pBIN20-35S-NPP

Fig. 3: Isolation of the 70-kDa NPPase by isoelectric focusing (IEF) in a negative gel:

a) Corresponds to staining of the proteins after being separated by IEF, in relation to their isoelectric point (values shown at the top of the diagram). The cathode would be on the right and the anode on the left. A partially purified sample that contains 1 unit of AGPPase is applied to a plate of Ampholine PAG at a pH range 3.5-9.

b) Corresponds to a profile of NPPase activity. The NPPase activity of each fraction eluted from the IEF gel is measured.

c) Each fraction eluted from the IEF gel is submitted to SDS-PAGE and then staining with Coommassie-Blue. The fractions enriched with 70-kDa protein are indicated with an arrow and prove to be the most active enzymatically

Fig. 4: Comparison of amino acid sequences between the amino acid sequence deduced from the cDNA that codes for rice NPPase, and those deduced from the cDNA's that code for the PPD1 of Lupinus luteus (AJ421009) and unknown proteins of Arabidopsis (AY099570) and chickpea (AJ271664).

Fig. 5: Hydrolytic activities of ADPglucose in wild-type (WT) and 9 transgenic lines of potato that overexpress rice NPP.

Fig. 6: Western blot of 5 transgenic lines of potato that overexpress rice NPP. 50 micrograms of protein were loaded in each lane and were submitted to SDS-PAGE. After transfer to nitrocellulose filters, the NPP was immunodecorated specifically after using the

polyclonal antibody specific to NPPs obtained in the rabbit.